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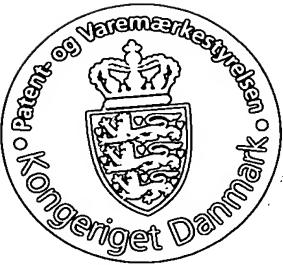
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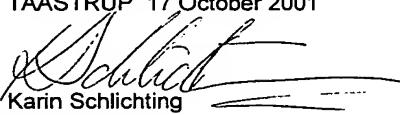
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PVS

PRODUCTION OF HETEROLOGOUS PROTEINS IN YEAST

The present invention relates to a method for production of heterologous proteins or intermediates thereof in yeast, in particular proteins of non-bacterial origin. The invention also relates

5 to DNA sequences and vector constructs for use in such method and transformed yeast cells.

BACKGROUND

Yeast is a widely used organism for production of desired proteins. Expression of foreign proteins in yeast is disclosed in e.g. EP 163,529, US 4,870,008 and US 5,618,676.

10 For commercial application high yields of the expressed proteins is important. A number of different factors will have an impact on the expression yield including the promoter and so-called upstream activating sequences. It is generally accepted that the best result is achieved when a promoter that is homologous to the host organism is used for expression of 15 both native and foreign genes in transformed host cells. Thus for expression of foreign genes in yeast a promoter associated with a yeast gene would be preferred. Several promoters useful for expression of heterologous proteins in yeast are known. The most commonly used yeast promoters include the *PGK*, *TPI*, *ADH2*, *PHO5*, *MFα1* and *GAP* promoters.

20 There is, however, a constant need for further promoters that have improved efficiency and the capability of being regulated for use in large scale thus enabling a high level of expression yield in commercial production.

SUMMARY OF THE INVENTION

25 The present invention provides a process for expression of heterologous non-bacterial proteins in yeast wherein a yeast promoter associated with the yeast *CIT1* gene or a functional part or variant of the *CIT1* promoter is used for expression of the heterologous gene.

30 In its broadest aspect the present invention is related to a process for making a heterologous, non-bacterial polypeptide in yeast or an intermediate therefore comprising (i) culturing a yeast strain comprising a polynucleotide sequence encoding the desired polypeptide or an intermediate therefore under suitable culture conditions for expression of said polypeptide, wherein the polynucleotide sequence encoding the desired polypeptide or its intermediate is under transcriptional control of a *CIT1* yeast promoter or a functional part or variant thereof, and (ii) isolation of the desired polypeptide or its intermediate.

In another aspect the present invention it is related to a polynucleotide construct comprising a polynucleotide sequence encoding a non-bacterial polypeptide or an intermediate therefore and the CIT1 yeast promoter or a functional part or variant of the CIT1 promoter.

5 In a further aspect, the present invention is related to a vector construct comprising in proper reading frame (a) the yeast CIT1 promoter or a functional part or variant thereof, (b) a polynucleotide sequence encoding a non-bacterial polypeptide or an intermediate therefore, (c) a suitable leader sequences and (d) possible a transcription terminator sequence.

10 In a further aspect, the present invention is related to yeast cells being transformed with the polynucleotide construct or vector of the invention.

In one embodiment the promoter sequence is at least 65% identical to the CIT1 yeast promoter sequence. In another embodiment the promoter sequence is at least 80, 85, 90 or 95% to the CIT1 yeast promoter sequence.

15 In a further embodiment the CIT1 yeast promoter variant has at least 75% sequence identity with a fragment of SEQ ID NO:1 which has the same length as the variant. In a still further embodiment the variant of the CIT1 promoter has at least 80, 85, 90 or 95% sequence identity with a fragment of SEQ ID NO:1 which has the same length as the variant.

20 The yeast promoter CIT1 directs the expression of the yeast citrate synthase gene C/T, vide Rosenkrantz et al, Curr Genet (1994) 25:185-195. The CIT1 promoter sequence is comprised in the sequence complementary to the sequence from position 2434 to position 3155 of GenBank accession number Z71616.

25 In another embodiment of the present invention the CIT1 promoter sequence is contained in SEQ ID NO:1 and will typically consist of all or part of the nucleotide sequence of SEQ ID NO:1. The promoter sequence used in the present invention may furthermore consist of all or part of the nucleotide sequence from 10 to 722 of SEQ ID NO:1. In a further embodiment the promoter consist of all or part of the nucleotide sequence from position 150 to 722 of SEQ ID NO:1 and in a still further embodiment the promoter may consist of all or part of the nucleotide sequence from position 150 to 530 of SEQ ID NO:1.

30 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows an example of a yeast plasmid called pMT742 (Egel-Mitani et al., Gene, 73, 1988, 99, 113-120). The plasmid contains an expression cassette comprising an EcoRI – XbaI fragment inserted into the plasmid between the transcription-promoter (located on a SalI – EcoRI fragment) and the transcription-terminator of the *S. cerevisiae* TPI gene.

Fig. 2 shows the 722 nucleotide DNA sequence containing the CIT1 promoter described in example 1. Above the sequence is shown the nucleotide changes introduced by oligonucleotides E102 and E103. The underlined sequences denote the restriction sites introduced by these changes. The nucleotide sequence corresponds to the complementary 5 sequence from position 2434 to position 3155 of GenBank accession number Z71616.

DETAILED DESCRIPTION

With "CIT1" yeast promoter is meant the natural or wild type yeast promoter sequence disclosed by Rosenkrantz et al, Curr Genet (1994) 25:185-195 or its complementary 10 sequence. It is well known in the art that not all part of a natural promoter sequence is necessary for efficient transcription. Resections may be made at the N-terminal end or at the C-terminal end or at both the N-terminal and C-terminal ends. Furthermore one or more nucleotides can be deleted within the promoter sequence. Also, a minor number of individual nucleotides may be changes to other nucleotides, e.g. to introduce a suitable restriction site. 15 Thus "a functional part or variant of the CIT1 promoter" in this context is intended to cover such modified forms of the natural CIT1 promoter sequence as long as the transcriptional promoter activity is not adversely affected. A retention of at least 75% of the transcriptional promoter activity compared to a similar construct where everything else is the same except for the promoter sequence will be preferred. The functional part or variant of the 20 CIT1 promoter should not have less than 65% and more typically not less than at least 70 to 95% of the transcriptional promoter activity of the unchanged promoter.

In the context of the present invention a variant of the CIT1 promoter will include any sequence obtained from the SEQ ID NO:1 by mutation, deletion, substitution, addition and/or modifications of one or more nucleotides as long as the promoter variant retains at least 65% 25 of its transcriptional promoter activity in the unmodified state.

Whether a functional part or variant of the CIT1 promoter has retained at least 65% of its transcriptional promoter activity in the unchanged form can be demonstrated in various ways for example by use of a reporter gene which's expression is detectable in a convenient way.

30 The promoter and its variants may be preceded by upstream activating sequences "USA" as well known in the art.

A coding sequence is under "transcriptional control" of a promoter when RNA polymerase binds to the transcription initiation sequence and transcribes the coding sequence into mRNA terminating at the transcription termination sequence and the mRNA is 35 then translated into the polypeptide encoded by the coding sequence.

By "in proper reading frame" is meant that the individual polynucleotide sequences are operably connected in such a way that the coding sequence is under the transcriptional control of the yeast recognized promoter and terminator sequences.

By "yeast-recognized" promoter and terminator sequences is meant regulatory sequences that are functional in yeast.

By "percent sequence identity" is meant percent identity between two nucleotide sequences when aligned and compared nucleotide to nucleotide. Methods for sequence alignment and for determination of the percent identity are well known in the art, see for example Smith T.F. and Waterman M.S. (1981) Identification of common molecular subsequences. *J. Mol. Biol.* 147:195-7.

"POT" is the *Schizosaccharomyces pombe* triose phosphate isomerase gene, and "TPI1" is the *S. cerevisiae* triose phosphate isomerase gene.

By "insulin precursor" is meant a single-chain polypeptide which by one or more subsequent chemical and/or enzymatic processes can be converted to a two-chain human insulin. The single-chain insulin precursor will contain correctly positioned disulphide bridges (three) as in human insulin.

By a "leader" is meant an amino acid sequence consisting of a pre-peptide (the signal peptide) and a pro-peptide.

The term "pro-peptide" means a polypeptide sequence whose function is to allow the expressed polypeptide to be directed from the endoplasmic reticulum to the Golgi apparatus and further to a secretory vesicle for secretion into the culture medium (i.e. exportation of the polypeptide across the cell wall or at least through the cellular membrane into the periplasmic space of the yeast cell). The pro-peptide may be the yeast α -factor pro-peptide, vide US 4,546,082 and 4,870,008. Alternatively, the pro-peptide may be a synthetic pro-peptide, which is to say a pro-peptide not found in nature. Suitable synthetic pro-peptides are those disclosed in US 5,395,922; 5,795,746; 5,162,498 and WO 98/32867. The pro-peptide will preferably contain an endopeptidase processing site at the C-terminal end, such as a Lys-Arg sequence or any functional analogue thereof.

The term "signal peptide" is understood to mean a pre-peptide which is present as an N-terminal sequence on the precursor form of a protein. The function of the signal peptide is to allow the heterologous protein to facilitate translocation into the endoplasmic reticulum. The signal peptide is normally cleaved off in the course of this process. The signal peptide may be heterologous or homologous to the yeast organism producing the protein. A number of signal peptides which may be used with the DNA construct of the invention including yeast aspartic protease 3 (YAP3) signal peptide or any functional analogue (Egel-Mitani et al.

(1990) YEAST 6:127-137) and the α -factor product of the *MF α 1* gene (Thorner (1981) in The Molecular Biology of the Yeast *Saccharomyces cerevisiae*, Strathern et al., eds., pp 143-180, Cold Spring Harbor Laboratory, NY).

The term "heterologous polypeptide or protein" means a protein or polypeptide
5 which is not produced by the host yeast organism in nature.

By "an intermediate" is meant a polypeptide or protein which can be converted into the desired final product by suitable chemical or physical means.

By "connecting peptide" or "C-peptide" is meant the connection moiety of the B-C-A
10 polypeptide sequence of a single chain preproinsulin-like molecule. Specifically, in the natural insulin chain, the C-peptide connects position 30 of the B chain and position 1 of the A chain.

By "insulin precursor" is meant a single-chain polypeptide which by one or more subsequent chemical and/or enzymatic processes can be converted into insulin. Insulin may be human insulin or a human insulin analogue having one or more mutations, substitutions, 15 deletions and or additions of the A and/or B amino acid chains relative to the human insulin molecule. The insulin analogues are preferably such wherein one or more of the naturally occurring amino acid residues, preferably one, two, or three of them, have been substituted by another codable amino acid residue.

With "desB30" or "B(1-29)" is meant a natural insulin B chain lacking the B30 amino 20 acid residue, "A(1-21)" means the natural insulin A chain

With "MI3" is meant an single chain insulin precursor of the B(1-29) chain linked to the A(1-21) chain by a short peptide bridge AlaAlaLys.

The polynucleotide sequence of the invention may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by Beaucage et al. 25 (1981) Tetrahedron Letters 22:1859-1869, or the method described by Matthes et al. (1984) EMBO Journal 3:801-805. According to the phosphoamidite method, oligonucleotides are synthesized, for example, in an automatic DNA synthesizer, purified, duplexed and ligated to form the synthetic DNA construct. A currently preferred way of preparing the DNA construct is by polymerase chain reaction (PCR).

30 The polynucleotide sequence of the invention may also be of mixed genomic, cDNA, and synthetic origin. For example, a genomic or cDNA sequence encoding a leader peptide may be joined to a genomic or cDNA sequence encoding the A and B chains, after which the DNA sequence may be modified at a site by inserting synthetic oligonucleotides encoding the desired amino acid sequence for homologous recombination in accordance with well-known

procedures or preferably generating the desired sequence by PCR using suitable oligonucleotides.

The invention encompasses a vector which is capable of replicating in yeast and which carries a polynucleotide sequence encoding the desired polypeptide. The recombinant vector

5 may be an autonomously replicating vector, i.e., a vector which exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extra-chromosomal element, a mini-chromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Examples of sequences which enable the vector to replicate in yeast are the yeast plasmid 2 μ m replication genes

10 REP 1-3 and origin of replication.

Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a

15 transposon may be used. The vector may be linear or closed circular plasmids and will preferably contain an element(s) that permits stable integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.

The vectors of the present invention preferably contain one or more selectable markers which permit easy selection of transformed cells. A selectable marker is a gene the product

20 of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. A preferred selectable marker for yeast is the *Schizosaccharomyces pombe* TPI gene (Russell (1985) Gene 40:125-130).

In the vector, the polynucleotide sequence is operably connected to the promoter sequence of the invention. The promoter sequence may be preceded by up stream enhancer sequences.

30 The invention encompasses a recombinant expression vector which is capable of replicating in yeast and which carries a polynucleotide construct encoding the desired product or an intermediate therefore. The recombinant expression vector may be any vector which is capable of replicating in yeast organisms. In the vector, the polynucleotide sequence should be operably connected to the selected promoter sequence. The polynucleotide construct of

the invention may also be operably connected to a suitable terminator, for example the TPI terminator (Alber et al. (1982) *J. Mol. Appl. Genet.* 1:419-434).

The procedures used to ligate the polynucleotide constructs of the invention, the promoter and the terminator, respectively, and to insert them into suitable yeast vectors containing the information necessary for yeast replication, are well known to persons skilled in the art. It will be understood that the vector may be constructed either by first preparing a DNA construct containing the entire DNA sequence of the invention, and subsequently inserting this fragment into a suitable expression vector, or by sequentially inserting DNA fragments containing genetic information for the individual elements (such as the signal, leader, promoter and the desired product) followed by ligation.

The yeast organism used in the process of the invention may be any suitable yeast organism which, on cultivation, produces large amounts of the insulin precursor and insulin precursor analogs of the invention. Examples of suitable yeast organisms may be strains selected from the yeast species *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*, *Schizosaccharomyces pombe*, *Saccharomyces uvarum*, *Kluyveromyces lactis*, *Hansenula polymorpha*, *Pichia pastoris*, *Pichia methanolica*, *Pichia kluyveri*, *Yarrowia lipolytica*, *Candida* sp., *Candida utilis*, *Candida cacaoi*, *Geotrichum* sp., and *Geotrichum fermentans*.

The transformation of the yeast cells may for instance be effected by protoplast formation followed by transformation in a manner known *per se*. The medium used to cultivate the cells may be any conventional medium suitable for growing yeast organisms. The secreted heterologous protein, a significant proportion of which will be present in the medium in correctly processed form, may be recovered from the medium by conventional procedures including separating the yeast cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate or by ion exchange chromatography, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, affinity chromatography, or the like.

After secretion to the culture medium and recovery, the isolated polypeptide will be subjected to various in vitro procedures for example to remove a possible N-terminal extension sequence and/or other unwanted sequences in the expressed and secreted product. Thus the desired polypeptides, e.g. an insulin precursors or an insulin precursor analogue may be expressed with an N-terminal amino acid residue extension, as described in U.S. Patent No. 5,395,922, and European Patent No. 765,395A, both of which patents are herein specifically incorporated by reference. The N-terminal extension may be removed from the recovered insulin precursor or insulin precursor analogue by means of a proteolytic enzyme

which is specific for a basic amino acid (e.g., Lys) so that the terminal extension is cleaved off at the Lys residue. Examples of such proteolytic enzymes are trypsin or *Achromobacter lyticus* protease. If the insulin precursor or insulin precursor analogue comprise a connecting peptide such connecting peptide will be cleaved off by in vitro enzymatic conversion by means

5 of trypsin or a trypsin like enzyme or an *Achromobacter lyticus* protease. If the insulin pre-
cursor or insulin precursor analogue is expressed and secreted as single chain precursor
wherein the B29 amino acid residue is linked to the A1 amino acid residue by means of a
peptide bond or a connecting peptide such as an AlaAlaLys sequence (MI3) the insulin pre-
cursor is converted into insulin by enzymatic conversion with trypsin or *Achromobacter lyti-
cus* protease in the presence of an L-threonine ester followed by conversion of the threonine
ester of the insulin or insulin analogue into insulin or the insulin analogue by basic or acid
hydrolysis as described in US patent specification No. 4,343,898 or 4,916,212.

The present invention is described in further detail in the following examples that are
not in any way intended to limit the scope of the invention as claimed. The attached Figures
15 are meant to be considered as integral parts of the specification and description of the invention.
All references cited are herein specifically incorporated by reference for all that is de-
scribed therein.

EXAMPLES

General Procedures

Plasmids and DNA

All expressions plasmids are of the C-POT type, similar to those described in WO EP 171
142, which are characterized by containing the *Schizosaccharomyces pombe* triose phos-
phate isomerase gene (POT) for the purpose of plasmid selection and stabilization in *S. cer-
evisiae*. The plasmids furthermore contain the *S. cerevisiae* triose phosphate isomerase
terminator.

Yeast Strain and Transformation

S. cerevisiae strain MT663 (*MATa/MAT α pep4-3/pep4-3 HIS4/his4 tpi::LEU2/tpi::LEU2*
Cir⁺) was used as host strain for transformation. Strain MT663 was deposited in the
30 Deutsche Sammlung von Mikroorganismen und Zellkulturen in connection with filing WO
92/11378 and was given the deposit number DSM 6278. Transformation of MT663 was con-
ducted as described in WO 98/01535.

Yeast Expression

Yeast strains harbouring plasmids as described above, were grown in YPD media
35 (Guthrie , C. & Fink, G.R., Eds., Guide to Yeast Genetics and Molecular Biology, Academic

Press, 1991). For each strain 2-4 individual 5 ml cultures were shaken at 30°C for 72 hours, with a final OD₆₀₀ of approximately 15-20. After centrifugation the supernatant was removed for quantitative analysis by which method the concentration of secreted heterologous product was measured. For insulin precursors the analysis was by HPLC by a method described by Snel, L. et al. *Chromatographia* 24 (1987) 329-332. For Sf-IBP an insulin binding assay was performed as described by Andersen, A.S. et al., *J. Biol. Chem.* 275 (2000) 16948-16953. For AMMEP an assay for proteolytical activity was performed as follows: An MI3 precursor with the extension EEAEEAEAK(SEQ ID NO:2) (described in Kjeldsen et al., *Gene* 170: 107-112, 1996) was incubated with supernatant from yKV333 or yEA284 overnight at 37°C. Subsequently the supernatants were analysed for EEAEEAEAK(SEQ ID NO:2)-extended MI3 or MI3 with a single N-terminal Lys residue (the product that results from AMMEP cleavage) by HPLC as described above. The ratio of Lys-MI3/EEAEEAEAK(SEQ ID NO:2)-MI3 was used as a measure for AMMEP expression level.

In table 1 the expression levels of the insulin precursor MI3, the N-terminal extended MI3, as well as Sf-IBP and AMMEP obtained by use of the CIT1 promoter are given as percentage of the level obtained with the corresponding constructs using the TPI1 promoter instead of the CIT1 promoter.

Example 1.

Construction of expression vector encoding an insulin precursor MI3 under expression control of the CIT1 promoter.

Figure 1 shows a yeast plasmid called pMT742 (Egel-Mitani et al., *Gene*, 73, 1988, 99. 113-120). The plasmid contains an expression cassette comprising an EcoRI - XbaI fragment inserted into the plasmid between the transcription-promoter (located on a SalI - EcoRI fragment) and the transcription-terminator of the *S. cerevisiae* TPI gene.

In plasmid pMT742 the EcoRI - XbaI fragment encodes a fusion product composed of the MFα1 pre-pro leader, a Lys-Arg cleavage site for the dibasic processing endopeptidase KEX2, and the single-chain mini-insulin precursor MI3. In order to construct a plasmid where the promoter from the TPI gene has been replaced by the promoter from the CIT1 gene of *S. cerevisiae*, the following steps were performed using standard molecular biology techniques (e.g. Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning: A laboratory Manual*, Cold Spring Harbour Laboratory Press, New York, 1989).

A 722 bp DNA fragment containing the CIT1 promoter was amplified with PCR from genomic *S. cerevisiae* DNA (from strain MT663) using oligonucleotides E102 (5'-TAT AAG 35 AGA GGT CGA CTT ATT GTT GTC AGC CCA ATG ATT C-3') (SEQ ID No: 3) and E103

(5'-TAT CGC TGA CGA ATT CGT AAA TAG TAT TAT ATT GCT ATA TGT-3') (SEQ ID NO:4). E102 has been designed to introduce a *Sall* restriction site, and E103 an *EcoRI* restriction site, into the amplified DNA fragment. After digestion with *Sall* and *EcoRI* the CIT1 promoter can thus be cloned as a *Sall* - *EcoRI* fragment.

5 To facilitate the cloning, the *Apal* - *EcoRI* of pMT742 containing the TPI promoter from *S. cerevisiae* was subcloned into a pBluescriptII-SK (Stratagene) *Apal* - *EcoRI* vector fragment. The resulting plasmid pEA222 was digested with *Sall* and *EcoRI* and the vector fragment was ligated to the *Sall*/*EcoRI* DNA fragment containing the CIT1 promoter. The resulting plasmid pSurf/CIT was digested with *Ncol* and *EcoRI* and the DNA fragment containing the CIT1 promoter was ligated to the *Ncol*/*XbaI* vector fragment and the *EcoRI*/*XbaI* fragment from pMT742 resulting in the final plasmid pEA268.

10

The expression plasmid was propagated in *E. coli*, grown in the presence of ampicillin and isolated using standard techniques (Sambrook et al., 1989). The plasmid DNA was checked for insert by appropriate restriction nucleases (e.g. *EcoRI*, *Ncol*, *Sall*, *XbaI*) and was shown by sequence analysis to contain the proper sequence of the CIT1 promoter.

15 The plasmid pEA268 was transformed into *S. cerevisiae* strain MT663. Yeast transformants harbouring plasmid pEA268 were selected by glucose utilization as carbon source on YPD (1% yeast extract, 2% peptone, 2% glucose) agar (2%) plates and the resulting strain was named yEA268. The control strain MT742 is MT633 transformed with pMT742.

20

Example 2

Construction of expression vector encoding an N-terminal extended insulin precursor MI3 under expression control of the CIT1 promoter

25 The EcoRI/XbaI DNA fragment containing the expression cassette from plasmid pAK729 (described in WO 97/22706) encodes a fusion product composed of a signal peptide followed by a synthetic leader, a Lys-Arg cleavage site for the dibasic processing endopeptidase KEX2, a spacer and processing site (EEAEPK) (SEQ ID NO:5) and the single-chain mini-insulin precursor MI3. This fragment was ligated to the *Ncol*/*XbaI* and the *Ncol*/*EcoRI* fragment (containing the CIT1 promoter) from pEA268 resulting in the final plasmid pEA274. The plasmids were propagated in *E. coli*, isolated and checked for the correct insert as in example 1.

30

The plasmids pAK729 and pEA274 were transformed into *S. cerevisiae* strain MT663 and transformants selected as in example 1. The resulting yeast strains were named yAK729 and yEA274, respectively.

Example 3**Construction of expression vectors for Sf-IBP containing the TPI or CIT1 promoter**

Spodoptera frugiperda (fall armyworm) insulin-like peptide binding protein has been described by Andersen, A.S. et al., J. Biol. Chem. 275 (2000) 16948-16953 and the DNA sequence for the gene has been deposited in GenBank with accession number AF236641.

Oligonucleotides were designed that allowed PCR amplification of a DNA fragment encoding amino acids 23 to 265 of Sf-IBP furnished with an N-terminal extension having the amino acid sequence EEAEPK. Using a combination of PCR and overlap PCR, followed by isolation and cloning by standard methods (Horton et al., Gene 77:61-68, 1989, Sambrook et al., 1989) an expression vector pEA263 containing an expression cassette encoding SP-leader-KR-Ext-Sf-IBP was obtained. The EcoRI/Nhel fragment from plasmid pEA263 containing the expression cassette was ligated to the Ncol/Nhel and the Ncol/EcoRI fragment (containing the CIT1 promoter) from pEA268 resulting in the final plasmid pEA286.

The plasmids were propagated in *E. coli*, isolated and checked for correct insert as in example 1. Plasmids pEA263 and pEA286 were transformed into *S. cerevisiae* strain MT663 and transformants selected as described in example 1. The resulting yeast strains were named yEA263 and yEA286, respectively.

Example 4**Construction of expression vector for AMMEP**

The gene for the *Armillaria mellea* endometallopeptidase has been cloned by Vad, K and Thim, L. and the nucleotide sequence has been deposited in GenBank with accession number AJ238718. Oligonucleotides containing EcoRI and XbaI restriction sites were designed to PCR-amplify the entire coding sequence. After isolation and digestion with EcoRI and XbaI, the EcoRI/XbaI DNA fragment containing the entire AMMEP coding sequence was ligated to the Ncol/XbaI vector fragment and the Ncol/EcoRI fragment containing the TPI promoter (both from pMT742) resulting in the final plasmid pKV333.

The EcoRI/XbaI fragment from plasmid pKV333 containing the expression cassette for AMMEP was ligated to the Ncol/XbaI and the Ncol/EcoRI fragment (containing the CIT1 promoter) from pEA268 resulting in the final plasmid pEA284.

The plasmids were propagated in *E. coli*, isolated and checked for insert as in example 1. Plasmids pKV333 and pEA284 were transformed into *S. cerevisiae* strain MT663 and transformants selected as in example 1. The resulting yeast strains were named yKV333 and yEA284, respectively.

Table 1 shows the increase in yield by use of the CIT1 promoter compared to the TPI promoter. It appears from the table that use of the CIT1 promoter gives from 50 to more tan 100% increase in expressed and secreted product.

10

Table 1

Heterologous Product	Relative expression level compared to control
MI3 (yEA268/MT742)	142 %
MI3 with N-terminal e tension SEQ ID NO:2 (yEA274/yAK729)	180 %
Sf-IBP (yEA286/yEA263)	226 %
AMMEP (yEA284/yKV333)	190 %

What we claim is:

1. A process for making a heterologous, non-bacterial polypeptide or an intermediate therefore in yeast comprising (i) culturing a yeast strain comprising a polynucleotide sequence encoding the desired polypeptide or an intermediate therefore under suitable culture conditions, wherein the polynucleotide sequence encoding the desired polypeptide or its intermediate is under transcriptional control of a yeast CIT1 promoter or a functional part or variant thereof; and (ii) isolating the expressed product.
5
2. A process according to claim 1 wherein the promoter is at least 65%, 70%, 80% or 85% identical to the yeast CIT1 promoter.
10
3. A process according to claim 1, wherein the promoter is at least 90% identical to the CIT1 promoter.
4. A process according to claim 1, wherein the promoter is at least 95% identical to the CIT1 promoter.
15
5. A process according to claim 1, wherein the promoter consists of all or part of the nucleotide sequence of SEQ ID NO:1.
6. A process according to claim 1, wherein the promoter consists of all or part of the nucleotide sequence from 10 to 722 of SEQ ID NO:1.
7. A process according to claim 1 wherein the promoter consists of all or part of the nucleotide sequence from position 150 to 722 of SEQ ID NO:1.
20
8. A process according to claim 1 wherein the promoter consists of all or part of the nucleotide sequence from position 150 to 530 of SEQ ID NO:1.
9. A process according to claim 1 wherein the variant of the CIT1 promoter has at least 75% sequence identity with a fragment of SEQ ID NO:1 which has the same length as the variant.
25
10. A process according to claim 1 wherein the variant of the CIT1 promoter has at least 80, 85, 90 or 95% sequence identity with a fragment of SEQ ID NO:1 which has the same length as the variant.
11. A process according to claim 1, wherein the expressed product is isolated from the culture medium.
30
12. A polynucleotide construct comprising a polynucleotide sequence encoding a non-bacterial polypeptide or an intermediate therefore and a DNA sequence encoding a CIT1 yeast promoter or a functional part or variant thereof.
13. A polynucleotide construct according to claim 12, wherein the promoter is at least 65%, 70%, 80% or 85% identical to the CIT1 promoter.
35

14. A polynucleotide construct according to claim 12, wherein the promoter is at least 90% identical to the CIT1 promoter.
15. A polynucleotide construct according to claim 12, wherein the promoter is at least 95% identical to the CIT1 promoter.
- 5 16. A polynucleotide construct according to claim 12, wherein the promoter consists of all or part of the nucleotide sequence of SEQ ID NO:1.
17. A polynucleotide construct according to claim 12, wherein the promoter consists of all or part of the nucleotide sequence from 10 to 722 of SEQ ID NO:1.
- 10 18. A polynucleotide construct according to claim 12, wherein the promoter consists of all or part of the nucleotide sequence from position 150 to 722 of SEQ ID NO:1.
19. A polynucleotide construct according to claim 10, wherein the promoter consists of all or part of the nucleotide sequence from position 150 to 530 of SEQ ID NO:1.
- 15 20. A polynucleotide construct according to claim 12, wherein the variant of the CIT1 promoter has at least 75% sequence identity with a fragment of SEQ ID NO:1 which has the same length as the variant.
21. A polynucleotide construct according to claim 12, wherein the variant of the CIT1 promoter has at least 80, 85, 90 or 95% sequence identity with a fragment of SEQ ID NO:1 which has the same length as the variant.
- 20 22. A polynucleotide construct according to claim 12 furthermore comprising a leader sequence for secretion of the expressed polypeptide.
23. Yeast expression vector comprising in proper reading frame (a) a polynucleotide sequence comprising a CIT1 yeast promoter or a functional part or variant thereof as disclosed in any of claims 1-12, (b) a polynucleotide sequence encoding a non-bacterial polypeptide or an intermediate therefore, (c) a suitable leader sequence and (d) a possible transcription terminator sequence.
- 25 24. Yeast cells transformed with a polynucleotide construct according to claim 12 to 22.
21. Yeast cells transformed with a vector according to claim 23.